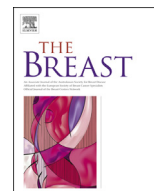


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## Original article

## Incorporating microarray assessment of HER2 status in clinical practice supports individualised therapy in early-stage breast cancer

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## ABSTRACT

Accurate determination of human epidermal growth factor receptor-2 (HER2) status is essential for optimal selection of breast cancer patients for gene targeted therapy. The analytical performance of microarray analysis using TargetPrint for assessment of HER2 status was evaluated in 138 breast tumours, including 41 fresh and 97 formalin-fixed paraffin embedded (FFPE) specimens. Reflex testing using immunohistochemistry/in situ hybridization (IHC/ISH) in four discordant cases confirmed the TargetPrint results, achieving 100% agreement regardless of whether fresh tissue or FFPE specimens were used. One equivocal IHC/ISH case was classified as HER2-positive based on the microarray result. The proven clinical utility in resolving equivocal and borderline cases justifies modification of the testing algorithm under these circumstances, to obtain a definitive positive or negative test result with the use of microarrays. Determination of HER2 status across three assay platforms facilitated improved quality assurance and led to a higher level of confidence on which to base treatment decisions.

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## Introduction

Overexpression of the human epidermal growth factor receptor-2 (HER2) occurs in 15–20% of all invasive breast cancers. Quantification of HER2 status plays an integral role in breast cancer prognostication and prediction of the response to HER2-targeted therapies, shown to result in a 30–50% improvement in disease-free and overall survival when combined with chemotherapy [1]. Assessment of HER2 status is therefore recommended in all patients with invasive breast cancer using immunohistochemistry (IHC) [2]. However, up to 20% of test results may be inaccurate, especially where testing is not centralised.

In an attempt to reduce variability in HER2 testing, the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommended that laboratories should demonstrate high concordance when comparing their results of HER2 testing with other validated HER2 tests [2]. Adoption of international external quality control measures improved the reliability and standardization of IHC HER2 testing. Fluorescence in-situ hybridisation (FISH) should be performed routinely in equivocal IHC 2+ cases, but small tumour size may be a limiting factor. Complete depletion of the invasive component of the tumour may occur and a high degree of discordance has been reported between different laboratories using FISH [3]. Interpretation of IHC/FISH results may be particularly challenging in cases with tumour heterogeneity or chromosome 17 polysomy. According to the ASCO/CAP revised criteria reflex testing should be performed in equivocal cases using an alternative assay. HER2 equivocal results and variability in

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reporting definitions of the optimal ranges for both IHC and FISH was identified as a gap in the literature as the decision to treat is by nature dichotomous (yes or no). The need for obtaining optimal test results warrants the development of novel methods that may be applied in conjunction with standard pathology to provide a definitive guidance in HER2 targeted therapy.

While determination of HER2 status based on mRNA expression levels has already been introduced into clinical practice, the ASCO/CAP Update Committee is of the opinion that there is insufficient evidence to support the use of genomic tests for this purpose [2]. Reverse transcriptase polymerase chain reaction (RT-PCR) is indeed considered unsuitable for determination of HER2 status [4]. This limitation was highlighted by concerns over the value of assessing HER2 status as part of the RT-PCR method used for the 21-gene Oncotype DX assay, which led to inappropriate HER2 targeted treatment in some patients [5,6]. Determination of HER2 status using multi-gene profiling tests is therefore not recommended due to potential clinical implications of inaccurate results and the impact on cost-effectiveness [6,7]. However, whether this restriction also applies to microarray-based multi-gene assays remains unclear, especially when formalin fixed paraffin embedded (FFPE) tumour tissue is used. Sapino et al. [8] successfully transferred microarray analysis using the 70-gene MammaPrint test from the initial use of fresh tumour to the more convenient use of FFPE tissue; however, a direct comparison between protein expression (IHC) and microarray-based mRNA expression (TargetPrint) for assessment of HER2 status using FFPE specimens has not been reported previously in relation to reflex testing in discordant cases.

HER2 status is reported as a separate read-out (TargetPrint) from the versatile MammaPrint microarray that enables the identification of a subgroup of low-risk patients with HER2-positive breast cancer [9]. In patients classified as low-risk according to the 70-gene MammaPrint profile, chemotherapy can be safely avoided without compromising long-term clinical outcome [10]. MammaPrint has been available in South Africa since 2007, and in 2009 local referral guidelines have been adopted to improve cost-effectiveness [11]. These guidelines referred to as the MammaPrint prescreen algorithm (MPA), exclude hormone receptor negative and HER2 positive patients for reimbursement by medical aid funders. This resulted in a highly selected study population of HER2 negative cases, as well as IHC/ISH equivocal and borderline HER2-positive cases that could be further assessed in relation to the clinical dilemma presented under these circumstances.

The aim of the study was to determine the level of agreement between HER2 status based on TargetPrint compared to standard IHC/FISH performed at various local laboratories in South Africa. To our knowledge, the clinical utility of TargetPrint using FFPE tumour specimens for the majority of samples tested has not previously been investigated at the interface between the laboratory and the clinic. Analytical validation of TargetPrint in the South African population is important due to the significant impact of HER2 status on treatment decision-making. Quality assurance may also be improved as a result of this study by obtaining a second opinion of HER2 status based on objective microarray analysis.

## Subjects and methods

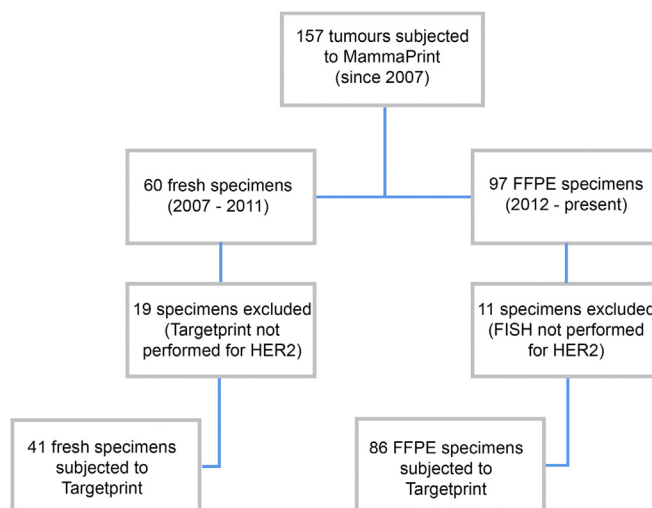
A central genomics database was established to collect data from South African breast cancer patients using an ethically approved protocol. MammaPrint became commercially available in South Africa after approval of the test by the Food and Drug Administration (FDA) in 2007. TargetPrint was added as a separate read-out from the MammaPrint microarray platform from 2009, providing quantitative estrogen receptor (ER), progesterone receptor (PR) and HER2 status.

## Study population

From a total of 157 early-stage breast cancer tumours successfully analysed using the 70-gene MammaPrint microarray profiler, 19 cases were excluded from this study as TargetPrint was not performed prior to 2009. Of the remaining 138 tumours, RNA was extracted for microarray analysis from 41 fresh tumours and 97 FFPE tissue biopsies. The TargetPrint mRNA expression levels were compared with routinely performed IHC and in situ hybridisation (ISH) assessments of HER2 status. As outlined in Fig. 1, HER2 assessments based on protein expression (IHC), DNA amplification (FISH) and mRNA expression (TargetPrint) were performed in a total of 127 samples. A FISH result was not available for 11 of the 138 specimens subjected to TargetPrint.

## Analysis of HER2 status using immunohistochemistry (IHC)

Assessment of HER2 status by protein expression using IHC was performed during routine analysis according to local laboratory procedures. Resected surgical specimens or needle core biopsies were fixed in 10% neutral buffered formalin for 6–48 h as specified in the 2013 ASCO/CAP guidelines for HER2 testing. Tissue blocks were processed according to analytically validated protocols and 3 µm paraffin embedded tissue sections were mounted on Histo-Bond® (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) positively charged slides. Slides were baked at 60 °C for 30 min in an incubator. Dewaxing and staining of slides were performed on the Leica Bond III™ automated IHC/ISH instrument (Leica Biosystems Pty Ltd, Melbourne, Australia) using the Bond Polymer Refine Detection kit (Cat.DS9800) with diaminobenzidine (DAB) chromogen. The primary antibody, Novocastra™ HER2 (Leica Biosystems, Newcastle, UK) clone 10A7 (Cat. NCL-L-CBE) targeting the external domain of the cell membrane, was diluted 1:50 using Bond™ Primary antibody diluent (Cat.AR9352). Pre-treatment was performed at a pH of 6 for 10 min at room temperature using the Bond™ Epitope Retrieval 1 solution (Cat. AR9961). The primary antibody was incubated for 20 min at room temperature. Positive control sections containing known positive 2+ and 3+ tumour sections were added to each slide for quality control purposes. Non-specific staining for each detection kit was performed omitting the primary antibody. The manufacturer's diaminobenzidine (DAB) detection kit was used that includes the biotin-free polymerase



**Fig. 1.** Selection of 41 fresh and 97 FFPE tumour specimens for comparative analysis of HER2 status between microarray analysis (TargetPrint) and standard IHC/FISH.

horseradish peroxidase (HRP)-antibody conjugate system for the detection of tissue-bond mouse and rabbit IgG and some mouse IgM primary antibodies, with DAB as the localisation reagents. Positive c-erb B2 staining of membranes were evaluated and scored using the 2013 ASCO/CAP guidelines recommendations for HER2 testing in breast cancer. External quality control and assessment of IHC protocols were validated by submission of in-house and UKNEQAS stained slides to UKNEQAS (United Kingdom-National External Quality Assessment scheme) for evaluation of IHC protocols in the reference laboratory.

#### Analysis of HER2 status using in situ hybridisation

FFPE specimens were investigated for HER2 gene amplification using the Vysis PathVysion HER-2 DNA probe kit [12]. Locus Specific HER2 and chromosome 17 control FISH probes were used to evaluate the distribution ratio of HER2 to control chromosome 17 centromeric probe signals (HER2/CEP17). FISH was generally not performed in IHC-negative (0/1+) cases, except when discrepancies were reported. In cases where IHC 2+ or 3+ scoring of HER2 was reported, FISH was performed on request of the treating oncologist. Eleven of the IHC 2+ tumours had no further FISH testing due to the above-mentioned limitations and treatment or cost considerations. All IHC 3+ tumours were also tested by FISH as local medical insurers only fund anti-HER2 therapy based on a positive FISH test. For the purpose of this study the term ratio always applies to the HER2/CEP17 ratio, as defined in the ASCO/CAP guidelines [2].

#### Analysis of HER2 status using microarray analysis (TargetPrint)

Microarray analysis was performed at the Agendia Laboratory in the Netherlands as previously described, using a pathology-supported genetic testing strategy implemented in 2009 [11]. This approach largely excluded HER2-positive cases from our study population based on the MPA developed in South Africa as a cost-saving strategy, as risk stratification is provided over and above assessment of ER, PR and HER2 status already routinely performed in all breast cancer patients using less expensive IHC/FISH methodology. A minimum of 30% tumour cell percentage was accepted for microarray analysis in accordance with FDA requirements, using six replicate measurements of HER2 mRNA expression consolidated in a single score considered positive for expression when a value of  $\geq 0$  was achieved [13].

#### Comparative analysis of HER-2 status

HER2 status determined in 138 tumour samples (41 fresh biopsies and 97 FFPE specimens) were compared between locally performed IHC/FISH results and microarray readout using TargetPrint. Concordance was regarded as the agreement between different testing methods concerning both positive and negative results. If different tests provided results which were negative versus equivocal or positive versus equivocal, these results were not regarded as discordant. Equivocal is not the same as discordant, but poses a clinical dilemma regarding treatment decisions for breast cancer patients with HER2 equivocal status.

#### Reflex testing

In cases where discordant results were observed between the initial routinely performed IHC/FISH tests and TargetPrint, repeat testing was performed on the same tumour tissue block used for TargetPrint. For further clarification in specific cases FISH was repeated on both the block originally used for IHC/FISH as well as

the FFPE tissue block afterwards used for TargetPrint, when discrepancies were reported from tests performed on different tissue blocks. Silver enhanced in-situ hybridization (SISH) was performed in one sample with equivocal HER2 status reported following IHC and FISH, to allow for quantitative scoring of the gene copy number. The initial FISH report of this patient was not available for further evaluation in this study. Assigning HER2 status was complicated by the fact that results reported prior to 2013 were indicated as equivocal when the FISH ratio ranged between 1.8 and 2.2. A ratio of 2 is currently used as the cut-off point, defining  $<2$  as HER2-negative and  $>2$  as HER-positive. Results are reported as provided in the reports available for evaluation and entered in the database at referral.

## Results

### Baseline characteristics

The baseline characteristics of 138 tumour specimens evaluated in his study are presented in relation to previous MammaPrint test results in Table 1.

#### Comparison of HER2 status determined by IHC and in situ hybridisation

FISH was performed on 24 (17.4%) of the 138 samples, based on IHC positive (3+) or equivocal (2+) results (Table 2). In the small group of IHC 3+ cases a high discordance rate of 57% (4/7) was observed in relation to positive FISH amplification of HER2 (ratio  $>2$ ). In the IHC 2+ group, 75% (12/16) of tumours were negative for HER2 amplification when assessed by FISH. Only one tumour reported as IHC 2+ remained FISH/SISH equivocal for HER2 status.

#### Comparison of HER2 status between IHC/FISH and TargetPrint

In four tumours TargetPrint HER2 status and the original IHC/FISH reports were discordant, with one case reported as HER2 negative by IHC. Resolution of an equivocal IHC/FISH testing report was also provided by TargetPrint in one sample. Reflex FISH/SISH testing at two different reference laboratories after sample

**Table 1**

Clinical characteristics and HER2 status presented in relation to the 70-gene MammaPrint microarray profile performed in 138 tumours of breast cancer patients.

	Total	70-gene profile	
		Lowrisk (%)	High-risk (%)
Total	138	84 (60.9)	54 (39.1)
Specimen			
Fresh	41 (29.7)	25 (61)	16 (39)
FFPE	97 (70.3)	59 (60.8)	38 (39.2)
Age (years), mean	53.1	54.8	50.6
$<36$	6 (4.3)	2 (33.3)	4 (66.7)
36–45	29 (21)	14 (48.3)	15 (51.7)
46–55	49 (35.5)	32 (65.3)	17 (34.7)
$>55$	54 (39.1)	36 (66.7)	18 (33.3)
Tumour Type			
Ductal	114 (82.6)	66 (57.9)	48 (42.1)
Lobular	21 (15.2)	15 (71.4)	6 (28.6)
Mucinous	3 (2.2)	3 (100)	0 (0)
Ductal Ca Grade			
1	31 (27.2)	21 (67.7)	10 (32.3)
2	62 (54.4)	36 (58.1)	26 (41.9)
3	20 (17.5)	8 (40)	12 (60)
N/A <sup>a</sup>	1 (0.9)	1 (100)	0

<sup>a</sup> N/A, not available.

**Table 2**  
Comparison of HER2 gene amplification determined by in situ hybridisation (ISH) and HER2 protein expression determined by immunohistochemistry in 24 tumour specimens.

FISH results	IHC results			Total
	Negative (%)	Equivocal (%)	Positive (%)	
	0/1+	2+	3+	
Positive	1(4.8%) <sup>a</sup>	3 (12.5%)	3 (12.5%)	7
Equivocal	0 (0%)	1 (4.2%)	0 (0%)	1 <sup>b</sup>
Negative	0 (0%)	12 (50%)	4 (16.7%)	16
Total	1	16	7	24

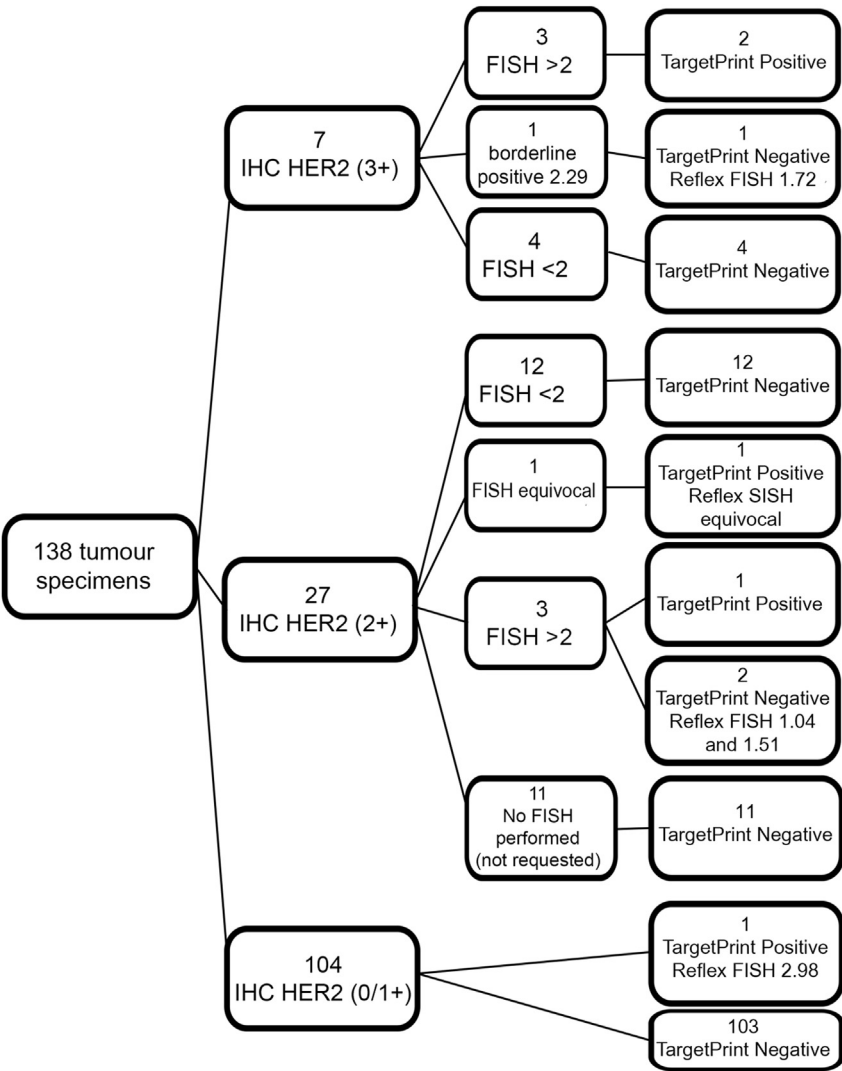
<sup>a</sup> FISH performed in IHC 0 case due to microarray result which confirmed the accuracy of Targetprint.  
<sup>b</sup> IHC 2+ remained equivocal on FISH and repeated SISH.

exchange confirmed the accuracy of the TargetPrint results when the same tissue block is used. As an equivocal result is not discordant, the correlation between IHC/ISH and microarray HER2 receptor status could be demonstrated with 100% agreement after reflex testing, as shown in Fig. 2. This flow diagram comparing IHC/FISH with TargetPrint HER2 assessment in the total number of 138 tumour specimens analysed includes the 24 samples compared in Table 2.

**Discussion**

The study was conducted at the interface between the laboratory and oncology practice in an attempt to address concerns over the accuracy of standard techniques used for determination of HER2 status in breast cancer patients. In addition to the known inconsistencies in IHC evaluation of HER2 and reported discrepancies in FISH results, an additional clinical dilemma is presented by equivocal FISH results [2]. The aim was to evaluate whether TargetPrint might provide additional benefit under these circumstances or potentially act as an alternative to FISH testing in patients referred for microarray analysis to further improve patient care and cost-effectiveness.

We demonstrated the added value provided by TargetPrint performed in conjunction with the 70-gene MammaPrint microarray in 138 tumour specimens. Risk reclassification of HER2 status based on TargetPrint following IHC/FISH reflex testing, supports the clinical utility of microarray-based gene profiling in breast cancer patients. Microarray-based assessment of HER2 status was highly reproducible and accurate, effectively reducing the number of false-positive and false-negative cases. A 100% concordance rate was obtained between IHC/FISH and TargetPrint results for HER2



**Fig. 2.** Flow diagram showing a comparison of HER2 status determined by IHC and in situ hybridisation, with HER2 status determined by TargetPrint following reflex testing as appropriate.



tumour status following reflex testing, irrespective of whether fresh surgical biopsies (41 samples) or FFPE specimens (97 samples) were used. This finding proved that use of FFPE tumour specimens provides a reliable source of RNA for microarray analysis of HER2 status, analytically validated for the first time in South African breast cancer patients using TargetPrint.

In spite of the fact that our series was limited by the large number of HER2 negative tumours due to pre-selection of the study population according to the MPA eligibility criteria applied in South Africa since 2009 [11], TargetPrint added important information in the five discordant/equivocal cases identified. The patient with the IHC-based HER2 negative tumour found to be HER2-positive according to TargetPrint, was given the option of trastuzumab treatment following confirmation by FISH. The three patients reported as HER2 positive according to the initial FISH result that were found to be negative following reflex FISH testing based on the HER2 negative TargetPrint results, could be spared the substantial expense and added toxicity of trastuzumab after careful evaluation of clinical and laboratory information obtained across three assay platforms. HER2-positive status assigned by TargetPrint in the patient with an equivocal ISH result facilitated treatment decision-making based on a binary value, which excluded the previous uncertainty of indeterminate ISH reporting. To our knowledge, this is the first study which initiated independent FISH retesting on the same tumour specimen used for TargetPrint when a discordant HER2 microarray result was obtained.

External quality control processes according to ASCO/CAP guidelines are in place in the laboratories where the IHC and FISH tests were performed. The high discordance rate observed between IHC 3+ and FISH amplification of HER2 observed in our study may be related to recent changes in IHC/FISH reporting definitions [2]. It may also be a reflection of our highly selected study cohort as a consequence of the MPA applied to determine eligibility for MammaPrint/TargetPrint in South Africa [11]. This approach resulted in only seven IHC 3+ tumours available for comparative analysis in this patient subgroup after excluding most HER2 positive tumours from microarray analysis based on previous FISH results. Importantly, as a result of this study, modification of the testing algorithm is justified to routinely include breast cancer patients with equivocal and borderline HER2-positive tumours for microarray gene profiling. This is an important consideration as some patients do not benefit from chemotherapy despite HER2 overexpression [14]. Future referral of patients with equivocal and borderline HER2-positive tumours for MammaPrint/TargetPrint microarray analysis may therefore be an important step towards chemotherapy-free therapy [9,14], which requires accurate assessment of HER2 status facilitated by microarray analysis.

Although all current HER2 testing techniques are limited in their ability to identify patients resistant to HER2-targeted therapy [15], retesting of IHC 0/1+ or FISH-negative cases is projected to be a cost-effective clinical strategy [16]. Uncertainty regarding the optimal cut-off value for HER2 testing in breast cancer however, remains a source of on-going debate [2,17]. In the analytical validation study of HER2 status using TargetPrint in the first 800 patients enrolled in the MINDACT (Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy) trial [13], tumours were considered HER2 positive when scored 3+ by IHC and/or amplified by FISH (ratio  $\geq 2$ ). However, Hanna et al. [18] supports the use of mean HER2 copy number rather than HER2:-CEP17 ratio to define HER2 positivity in cases where HER2 amplification could be masked by co-amplification of the centromere. Assigning HER2 status is further complicated by genetic heterogeneity and non-invasive cellular components co-existing in the same tumour, which may affect up to 40% of tumours when assessed according to the College of American Pathologists guideline.

Genetically heterogeneous tumours displaying HER2-amplified subclones have the potential to benefit from anti-HER2 therapy, but this has not yet been proven in clinical studies [18].

Some of the discrepant results reported for HER2 status in our study was related to the use of different FFPE tissue blocks for the initial FISH and TargetPrint tests, confirming the role of tumour heterogeneity in this context. Therefore, the FDA requirement of a minimum amount of 30% tumour for use of MammaPrint/TargetPrint is considered an important factor in the excellent performance of microarray-based determination of HER2 status. In a previous comparative study involving gene profiling a low percentage of invasive tumour in the tissue block used for Oncotype Dx was found in the majority (7/8, 88%) of discrepant cases reported between HER2 status using FISH versus RT-PCR [19]. In view of these findings and the results presented in this study, equivocal and borderline-positive HER2 status has become an important consideration when use of the Oncotype DX vs MammaPrint test is considered for chemotherapy selection. Rare categories of HER2 status not adequately covered by existing guidelines [2,20] are of particular relevance in this context and have the potential to change clinical practice as a result of the educational experience linked to monitoring of the quality of HER2 assessment, in real time, across three assay platforms (Dr Elizabeth Murray, personal communication).

HER2 is one of the key genes evaluated as part of the Oncotype DX recurrence score and the apparent incremental improvement in discrimination over the established prognostic factors is considered a limiting factor of this 21-gene RT-PCR assay. A large clinical validation study of Oncotype DX published in 2008 reported that the recurrence score was not predictive when determined in a subgroup of nearly 400 patients with HER2 negative tumours [21]. Similarly, performance of the current version of PREDICT, an online risk assessment tool that incorporates HER2 status in the model, is not considered to improve further with use of Oncotype DX to the same extent recently demonstrated for the 70-gene MammaPrint profile [22]. MammaPrint has the advantage that risk prediction for distant recurrence based on the 70 genes included in the test is calculated independent of HER2 status already determined routinely using less expensive IHC/FISH tests [9,11]. Compared to FISH and microarrays, Oncotype DX does not appear to accurately identify HER2-positive breast carcinomas [5,6], found to include a subpopulation that responds poorly to trastuzumab-based chemotherapy [23]. Concerns over sufficient evidence to base clinical decision-making concerning eligibility for trastuzumab therapy on multi-gene testing [5–7] do not apply to the advanced microarray technology evaluated in comparison with standard IHC/FISH in this study [24,25]. Recent recommendations caution against treatment decisions based on tumour grade alone or single biomarkers [24], given the potential of microarrays to capture the true biological profile of multiple genes regulating the functional activity of cancer pathways used as treatment targets [25].

## Conclusions

Analytical validation of MammaPrint [8] and TargetPrint (this study) on the basis of concordance results using FFPE tumour tissue is an important milestone in the era of personalised genomic medicine. Implementation of the MPA based on our pathology-supported genetic testing approach led to safe avoidance of chemotherapy in more than 60% of patients previously identified as low-risk for distant metastasis, irrespective of whether FFPE or fresh tumour biopsies were used [11]. According to the treating oncologists, no distant recurrence of disease or breast cancer-related deaths occurred to date in the MammaPrint low-risk group based on 8-year follow up data, as reported at the 9th

Applied Genetics Workshop registered (Ref. number 4133) as a Short Course at Stellenbosch University.

It is clear that the transfer from use of fresh to FFPE specimens in 2012 does not affect the microarray results negatively. TargetPrint improves quality assurance and provides a reliable ancillary method of assessing HER2 status in breast cancer. These findings suggest that in patients undergoing MammaPrint gene profiling, TargetPrint is a reliable alternative to standard assessment of HER2 at no additional cost. Modification of the testing algorithm to include equivocal and borderline HER-positive cases for referral of MammaPrint/TargetPrint is therefore justified based on the results presented in this study. Incorporation of TargetPrint using a pathology-supported genetic testing strategy improves clinical care by resolving borderline cases and also provides a second opinion on standard protein- and DNA-based HER2 testing.

### Conflict of interest statement

None declared.

### Disclosure

Prof MJ Kotze is a director and shareholder of Gknowmix (Pty) Ltd. that has developed a database tool for research translation under the auspices of the South African Medical Research Council. The authors have no other relevant affiliations or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

### Ethical approval

This study protocol was approved by the Ethics Research Committee of the University of Stellenbosch (reference number N09/06/166).

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